Relieving Mucus Flake Burden in Cystic Fibrosis

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I. Abstract

Dehydration of the mucus in the lung airway is a hallmark of cystic fibrosis (CF) airway disease. In CF, mucins—the key gel-forming polymeric component of mucus—are elevated in airway mucus. Roughly half of the mucins present in mucus are found in non-swelling gels termed "mucus flakes." Elevated mucus flake density is an indicator of CF disease and airflow obstruction. However, little is known about the factors that instruct flake formation or whether flakes can be therapeutically dissolved. Progress in understanding flakes is limited by the complexities of collecting and analyzing samples from human subjects.

We aim to identify the factors that promote mucus flake formation, allowing us to develop novel therapeutic approaches to dissolve them. A unique feature of our approach includes the *de novo* synthesis of mucus flakes from mucins isolated from human epithelial cells as a strategy to mimic flake structure and biochemical interactions. This strategy provides experimental tunability that will greatly enhance our understanding of flake formation and provide a platform to screen novel therapies to dissolve them. We tested the effects of various doses and combinations of a calcium chelator, reducing agent, surfactant, and known mucolytic as potential mucus flake dissolution agents. We found that the reducing agent, calcium chelator, surfactant, and cocktails of these reagents triggered significant flake swelling, resulting in increased water solubility, which may improve airway mucosal clearance. The calcium chelator and treatment cocktails also reduced flake number, which may reflect the dissolution of flakes, whereas the reducing agent and surfactant increased flake number and may demonstrate flake crumbling. This flake dissolution and crumbling may revert CF mucus to a healthy state.

This novel approach allows us to screen potential early treatments to delay the onset of lung disease in CF patients and to establish treatment effectiveness.

II. Introduction

The major macromolecular constituent of mucus is mucin, a type of heavily O-glycosylated protein and polymer "brush."[1] The mucus within the human lung airway is predominantly composed of MUC5B, a soluble, gel-forming mucin that is stabilized by disulfide bonds and hydrophobic interactions (Fig. 1).[2] MUC5B is secreted from secretory cells of the lung airway in the form of secretory granules.[3] Once secreted, MUC5B transitions from a condensed form—which is required for mucin packing within the secretory granule—to an expanded, linear conformation that is necessary for the formation of a functional mucus barrier.[3]

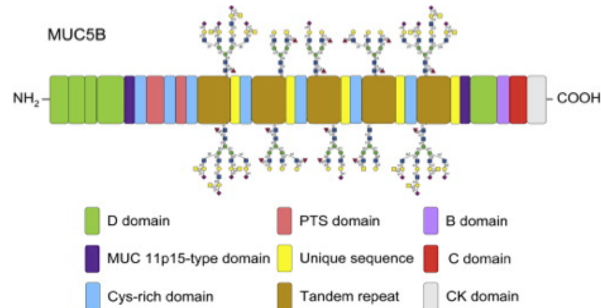

Figure 1. Schematic illustration of MUC5B structure. Relative sizes of domains are not drawn to scale[1]

Once the granule opens in the extracellular environment, crosslinking calcium ions between mucins are chelated by extracellular bicarbonate and are exchanged for sodium ions, which promotes mucin relaxation and expansion.[4] These ionic changes lead to significant shifts in osmotic pressure and an establishment of a local ion gradient, which results in an influx of water into the mucin network within mucus.[4] The influx of water subsequently hydrates airway mucus, reduces mucus viscoelasticity, and facilitates mucosal clearance.

Post-secretion expansion of MUC5B is sensitive to pH and calcium concentration changes in the mucus.[3] Specifically, deviations from the secretory granule's acidic pH (~pH 6) and high concentration calcium (~200 mM) environment impacted MUC5B conformation.[3] It has also been suggested that changes in pH, shifts in calcium concentration, and improper calcium chelation can lead to mucoviscidosis, which is also known as cystic fibrosis (CF) due to aberrant mucin unfolding post-secretion.[3]

CF is primarily caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which can change CFTR protein production or function.[5] As a result, abnormal transport of bicarbonate and chloride ions across the cell surface in the human airway can occur. This abnormal

transport can lead to symptoms, such as hyperconcentrated mucus, mucus accumulation, inflammation, and infection.[5] Viscous mucus accumulation within the airways drives airflow obstruction.[6] Current CF treatments involve DNase or hypertonic saline that reduce viscoelasticity by removing viscous host and bacteria-derived DNA and facilitating mucus hydration respectively.[4,6] The inhaled mucolytic N-acetyl cysteine (NAC) is also used as a CF treatment and works by targeting mucin disulfide bonds within airway mucus.[6]

Recently, in-homogenous domains of mucus have been discovered in preschool-aged pediatric CF patients who are enrolled in the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) program.[6] These in-homogenous domains of mucus termed “mucus flakes” form permanent hydrogels that are largely composed of MUC5B and DNA.[6] Though not specific to CF, the number of mucus flakes was found to be greater in CF patients than in non-CF (healthy patient) controls and associated with inflammatory and metabolic biomarkers of CF disease.[6] Due to the fact that mucus accumulation drives airflow obstruction, an increase in mucus flake size in addition to flake number may be associated with CF disease.[10]

As highlighted above, the relationship between flakes and mucins in CF and the human airways has been explored. However, the biochemical interactions that drive flake formation and treatments that aim to dissolve flakes have not been studied. Additionally, limited flake sample availability and the complexities of analyzing heterogenous mucus samples from human subjects pose additional challenges to study the formation and dissolution of flakes *in-vivo*.

A novel microfluidic device was previously designed and manufactured in the lab of Dr. Ronit Freeman at the University of North Carolina at Chapel Hill (Fig. 2).[10] This device efficiently creates artificial flakes from both calcium and mucin. Artificial flakes (Fig. 3C) were previously observed to form similar granular structures and morphologies as healthy (Fig. 3A) and CF (Fig. 3B)

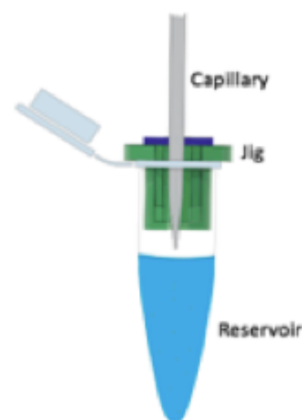

Figure 2. Fabricated Microfluidic Device for Mucus Flake Synthesis. Contains an Eppendorf with capillary for mucin and jig insertion for preparation of flake samples.

flakes.[10] As a result, this microfluidic device can be used to synthesize artificial *de novo* flakes to mimic healthy and CF flake structures. Using this approach, artificial flakes offer experimental tunability that provides an accessible platform to screen novel therapies for flake dissolution. Specifically, the importance of removing calcium, ionic bonds, disulfide bonds, and hydrophobic interactions from mucin polymers for flake disassembly remains largely unknown. Understanding how different treatments impact flake cross-linking interactions and cause degradation may allow us to test or improve CF therapeutics using an artificial mucus flake model.

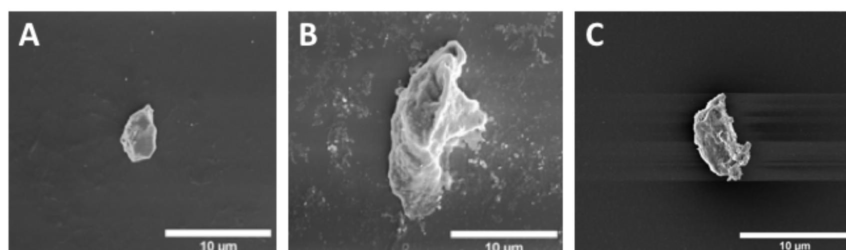

Figure 3. Comparison of Clinical and Artificial Mucus Flakes. Scanning electron microscopy (SEM) of healthy (A) and CF clinical (B) mucus flakes respectively. (C) Artificial mucus flake made using HBE MUC5B (10 mg/mL). These findings are unpublished.[10]

Here, we use this novel microfluidic device to investigate the changes in artificial flake size and number after treatment with the calcium chelator, EDTA, the reducing agent, dithiothreitol (DTT), and the surfactant, Tween-20. Furthermore, cocktails of different combinations of these reagents will be tested against artificial flakes. It is expected that flake exposure to these reagents will target and eliminate cross-linking calcium, disulfide bonds, hydrophobic interactions, and ionic bonds between mucin polymers within flakes, which may result in flake disassembly. Using the novel microfluidic device to synthesize *de novo* flakes to explore disassembly after reagent treatment, the diversity of flake structure and function can be recapitulated. More importantly, this approach can be used to explore therapeutic pathways to disassemble flakes and reduce the corresponding airway mucosal burden in CF.

III. Materials and Methods

Preparation of Synthesis Agent:

A 4 mM calcium chloride ($CaCl_2$) solution was prepared from a 500 mM stock solution. Human Bronchial Epithelial (HBE) mucin samples (10 mg/mL) were received from the lab of Dr. David Hill in the Marsico Lung Institute at the University of North Carolina at Chapel Hill where they had been purified from HBE cell cultures and stained with acid fuchsin. HBE mucin samples were stored at 4°C, rotating on a turntable.

Mucus Flake Preparation:

Glass capillaries were pulled (PE-22, Narishige). The diameters of the capillaries used were determined using a Nikon Microphot-FX microscope at 20X magnification. Capillaries used were measured to be within a range of 40 to 60 µm. Each capillary was washed with sterile water. The reservoir was filled with 4 mM $CaCl_2$. 5 µL of HBE mucin (10 mg/mL) was inserted into the capillary (Fig 2). Samples were centrifuged at 3000 RPM for 2 minutes and were subsequently resuspended to dissociate flakes.

Treatment Preparation and Exposure Assays:

Stock concentrations of 500 mM $CaCl_2$ and three reagents, including 100 mM dithiothreitol (DTT) (Sigma-Aldrich), 20 mM Tween-20 (Sigma-Aldrich), and 500 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) were made and stored at room temperature ($CaCl_2$, Tween-20, and EDTA) or at 4°C (DTT). The volume required for each reagent was calculated such that a final volume of 300 µL (100 µL of flakes in 4 mM $CaCl_2$ and 200 µL of reagent) was achieved per well in a Greiner Bio-One µClear™ Bottom 96-Well Polystyrene Microplate (Thermo Fisher Scientific) at each target concentration per reagent. Final reagent target concentrations of EDTA (20 mM, 50 mM, and 100 mM), DTT (1 mM, 10 mM, and 100 mM), and Tween-20 (0.1%, 1.0%, and 5.0%) were used for "single treatment" (treatment with only one reagent) exposure assays against prepared flake samples. Cocktails were made in a constant 4 mM $CaCl_2$ solution using the reagents at the final concentrations of: 10 mM DTT, 1% Tween-20, 3% hypertonic saline, and 50 mM EDTA. Cocktails of these reagents were categorized as "double cocktails,"

"triple cocktails," or "quadruple cocktails." Single treatments of EDTA, DTT, Tween-20, and saline were also tested in this cocktail sweep and will be referred to as "single sweep" treatments to differentiate from the single treatments tested outside of this cocktail sweep. 100 µL of prepared flakes were exposed to 200 µL of each treatment condition for 3 hours at 37°C, mixing consistently. Each treatment condition was completed in triplicate. Exposed flakes were immediately imaged using a GE INCell 2200 (GE Healthcare Life Sciences) after the 3 hours were complete.

<u>Mucus Flake Imaging:</u>

Prepared flake samples exposed to treatments were imaged at 20X using the GE INCell 2200 (GE Healthcare Life Sciences). The GE INCell 2200 included a solid-state illumination source and a sCMOS camera.

<u>Image J/Fiji Mucus Flake Analysis:</u>

Flake number and area were quantified using ImageJ/Fiji software. Background subtraction was done using a 50 pixel diameter rolling ball filter. Flake images were converted to 8-bit grayscale, and a "Max Entropy" threshold was applied. Flakes were filtered and analyzed using the bounds of 1 to infinity μm^2, circularity of 0.00-1.00, and the "exclude on edges" condition.

<u>Statistical Parameters and Tests:</u>

Significance of flake size and number after treatment were determined using a Kruskal-Wallis test (One Way ANOVA). A Gaussian distribution was not assumed (nonparametric test), and the mean rank of each treatment population was compared to the control (non-treated) population. Significance was determined using the parameters: non-significant (ns)>0.05, *<0.05, **<0.01, ***<0.001, ****<0.0001.

IV. Results

For all treatments, flakes were analyzed for two properties: (1) the size of the flake and (2) the flake number found relative to negative controls (non-treated). The size of flakes was determined by the number of pixels occupied per object and was normalized by the average size of experimental controls to determine the change in size. The number of flakes was determined by counting the number of flakes per image and normalizing it to the average number of flakes per image for the control. Ideally, after

treatment there would be no flakes remaining in the sample as elevated flake number is correlated with CF disease.[6] Barring complete dissolution, flakes would act as hydrogels and would most likely appear swollen as crosslinking sites (caused by hydrophobic collapse, calcium bridging, or other electrostatic interactions) are removed. We hypothesize that, much like the hyperconcentrated mucus in CF, less concentrated mucus flakes would be more readily cleared through mucociliary clearance.[7]

Reducing agent causes swelling and crumbling of mucus flakes

In mucus flakes, MUC5B mucin monomers are linked via disulfide bonds to create a mucin polymer.[2] Targeting these interactions with agents such as N-acetyl cysteine (NAC) have been shown as a therapy for CF; however, it is not known if therapeutic reagents that target these interactions dissolve flakes.

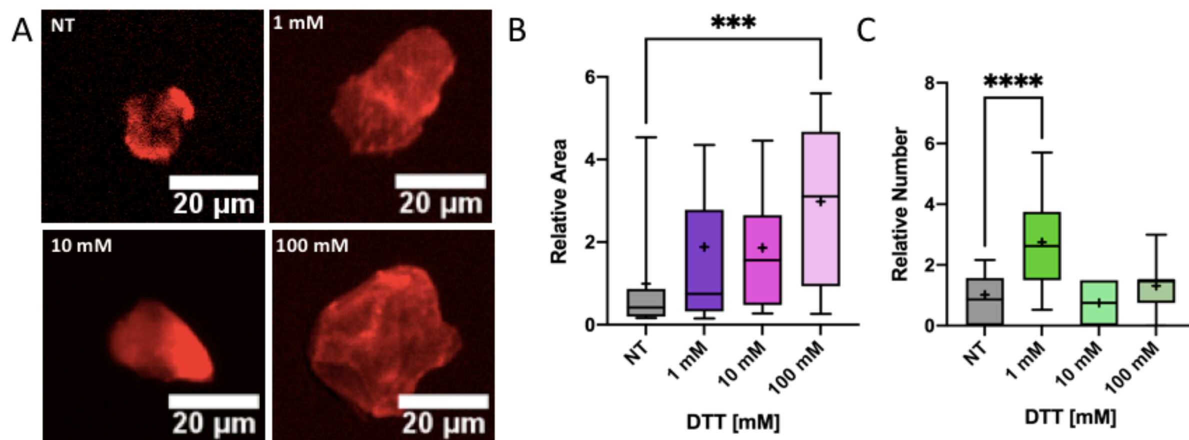

Figure 4. Mucus Flake Analysis after DTT Treatment (A) Representative non-treated (NT) mucus flakes and mucus flakes treated with 1 mM, 10 mM, and 100 mM DTT respectively. All mucus flakes were imaged at 20X. (B) Quantification of relative mucus flake size after treatment with 0 mM (NT), 1 mM, 10 mM, and 100 mM DTT. (C) Quantification of relative mucus flake number after treatment with 0 mM (NT), 1 mM, 10 mM, and 100 mM DTT. P-Values: *<0.05, **<0.01, ***<0.001, ****<0.0001. Plot whiskers measure 10-90 percentile error bars. Population average represented as "+" and median displayed as solid bar. Samples ran in triplicate where n=~100. A lower bound of 1μm^2 was used to quantify.

To eliminate the disulfide bonds that covalently link the mucin monomers together and subsequently break apart the mucin polymers within a flake, the reducing agent dithiothreitol (DTT) was added to flakes after synthesis. Flake samples were exposed to 1 mM, 10 mM, and 100 mM DTT for 3 hours at 37°C and subsequent relative flake area and number were measured.

Compared to the non-treated flake sample, the flake size after DTT addition observably increased at 20X magnification for the 1 mM and 100 mM DTT treatments, but no apparent size change was observed for the 10 mM treatment (Fig. 4A). Quantification of relative flake area confirmed that treatment with 100 mM DTT results in significantly increased flake size ($p<0.001$) (Fig. 4B*)*. While the size of flakes increased as a population for the 1 mM and 10 mM treatments, these size increases were not found to be statistically significant ($p>0.05$) (Fig. 4B).

The relative flake number increased significantly ($p<0.0001$) for the 1 mM DTT treatment (Fig. 4C). The change in relative flake size and number following DTT treatment post-synthesis confirms that DTT successfully targets the disulfide bonds within flakes at 100 mM (change in flake size) and 1 mM (change in flake relative number) DTT.

Calcium chelator drives mucus flake swelling and elimination

DTT acts as a reducing agent by breaking the disulfide bonds between the mucin monomers within a flake. In contrast, the calcium chelator, ethylenediaminetetraacetic acid (EDTA), binds to calcium between mucin polymers via four carboxylate and two amine groups, thereby sequestering calcium. It is unknown whether calcium chelators such as EDTA dissolve flakes by eliminating the interaction between positively charged calcium ions and negatively charged mucin polymers within flakes. Flakes were exposed to 20 mM, 50 mM, and 100 mM EDTA respectively for 3 hours at 37℃ and imaged at 20X magnification. This concentration range was determined based on literature EDTA concentrations previously used in CF models.[4]

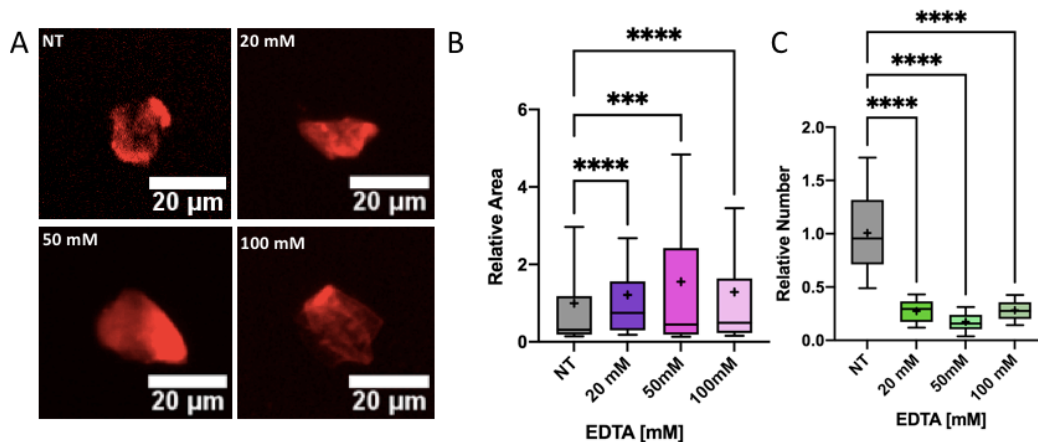

Figure 5. Mucus Flake Analysis after EDTA Treatment (A) Representative non-treated (NT) mucus flakes and mucus flakes treated with 20 mM, 50 mM, and 100 mM EDTA respectively. All mucus flakes were imaged at 20X. (B) Quantification of relative mucus flake size after treatment with 0 mM (NT), 20 mM, 50 mM, and 100 mM EDTA. (C) Quantification of relative mucus flake number after treatment with 0 mM (NT), 20 mM, 50 mM, and 100 mM EDTA. P-Values: *<0.05, **<0.01, ***<0.001, ****<0.0001. Plot whiskers measure 10-90 percentile error bars. Population average represented as ”+” and median displayed as solid bar. Samples ran in triplicate where n=~100. A lower bound of 1μm^2 was used to quantify.

A small increase in flake size at 50 mM and 100 mM EDTA was qualitatively observed relative to the non-treated flake sample; however, no apparent changes in flake size were observed for the 20 mM treatment (Fig. 5A).

Measurement of relative flake area confirmed that the flakes significantly increased in size following 50 mM and 100 mM EDTA exposure (p<0.001 and p<0.0001 respectively) (Fig. 5B). Interestingly, measurement of flake area also revealed a statistically significant flake size increase after 20 mM EDTA exposure (p<0.0001) (Fig. 5B). In addition to size changes in the flake population, the relative number of flakes significantly decreased after 20 mM, 50 mM, and 100 mM EDTA exposure (p<0.0001) (Fig. 5C). The changes in flake size and number following EDTA treatment confirm that the EDTA targets the calcium-mucin cross-linking interactions within flakes at 20 mM, 50 mM, and 100 mM EDTA.

Surfactant exposure drives mucus flake crumbling

To target the hydrophobic interactions that occur between the un-glycosylated protein regions at the N and/or C termini and cysteine-rich regions of HBE mucin polymers, the surfactant, Tween-20, was used as a treatment against flakes.[8] The flake exposure conditions to Tween-20 were identical to the previously described DTT and EDTA treatments. No changes in flake size at 0.1%, 1.0%, or 5.0% Tween-20 were observed at 20X magnification (Fig. 6A). Measurement of flake area confirmed that flakes did not significantly change in relative size following Tween-20 exposure ($p>0.05$) (Fig. 6B). In contrast, quantitative analysis revealed a significant increase in flake number for 0.1% ($p<0.0001$), 1.0% ($p<0.0001$), and 5.0% ($p<0.0001$) Tween-20 relative to the number of non-treated flakes (Fig. 6C). The change in flake number following Tween-20 treatment confirms that Tween-20 targets the hydrophobic interactions within mucus flakes at all concentrations.

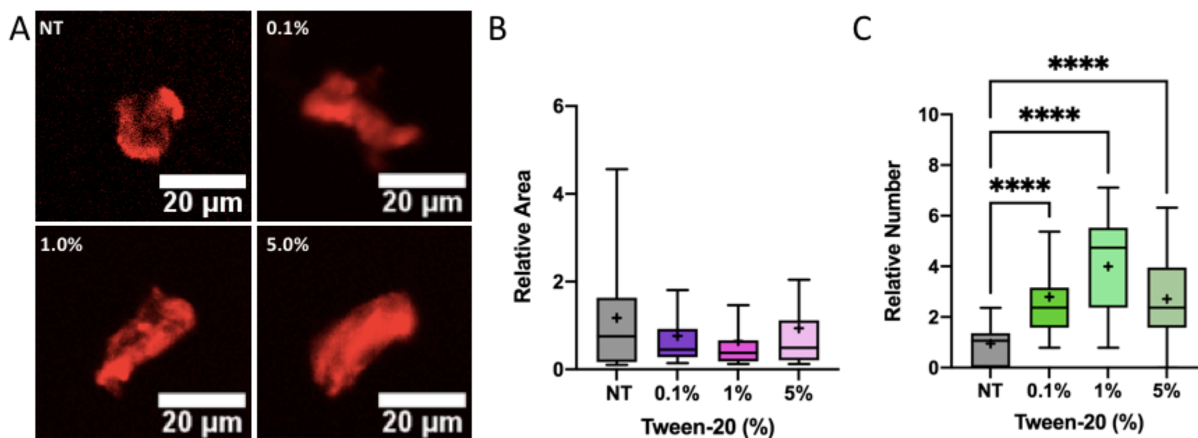

Figure 6. Mucus Flake Analysis after Tween-20 Treatment (A) Non-treated (NT) mucus flakes and mucus flakes treated with 0.1%, 1%, and 5% Tween-20 respectively. All mucus flakes were imaged at 20X. (B) Quantification of relative mucus flake size after treatment with 0% (NT), 0.1%, 1%, and 5% Tween-20. (C) Quantification of relative mucus flake number after treatment with 0% (NT), 0.1%, 1%, and 5% Tween-20. P-Values: *<0.05, **<0.01, ***<0.001, ****<0.0001. Plot whiskers measure 10-90 percentile error bars. Population average represented as "+" and median displayed as solid bar. Samples ran in triplicate where n=~100. A lower bound of $1 \mu m^2$ was used to quantify.

Changes in mucus flake size and number were evident after treatment with various cocktails

Flake exposure to various concentrations of single reagents, such as DTT, Tween-20, and EDTA have produced significant changes in relative flake size and number. Cocktails of EDTA, DTT, Tween-20, and hypertonic saline were tested, and changes in relative flake size and number were quantified to determine if combinations of reagents promote flake disassembly. Hypertonic saline is a known mucus-thinning and mucolytic nebulizer used in CF treatment.[4] Saline was used as an additional reagent in combination with EDTA, DTT, and Tween-20 to investigate flake disassembly. Various combinations of these reagents were tested, including "double cocktails," "triple cocktails," and a "quadruple cocktail." Single treatments of EDTA, DTT, Tween-20, and saline were also tested in this cocktail sweep and will be referred to as "single sweep" treatments to differentiate from the single treatment assays performed in figures 4-6. "Single sweep" treatments were similar to the EDTA, Tween-20, and DTT assays that were previously performed (Fig. 4-Fig. 6), except only a single concentration was tested for each reagent (50 mM EDTA, 1% Tween-20, 3% saline, and 10 mM DTT respectively) and a larger sample size was used ($n >> 100$). After flake exposure to the various cocktail combinations, relative flake size and number were analyzed and quantified.

In the "single sweep" treatments, relative number of flakes significantly decreased after treatment with 3% saline ($p<0.01$) (Fig. 7B). Relative flake size significantly increased after treatment with 10 mM DTT ($p<0.0001$) and 1% Tween-20 ($p<0.01$) (Fig. 7C).

"Double cocktail" treatments tested various two-reagent combinations against flake samples. After imaging at 20X, flake size appeared to decrease slightly for the "Saline + Tween-20" and "Saline + DTT" cocktails (Fig. 7A). Yet, no apparent changes in size were observed qualitatively for the "Saline + EDTA" cocktail (Fig. 7A). The relative flake size appeared to increase for the "EDTA + DTT," "EDTA+ Tween-20," and "DTT + Tween-20," cocktails (Fig. 7A). This increase was confirmed when flake relative areas were quantified and analyzed in comparison to the NT mucus flake sample (Fig. 7C). Specifically, relative flake size increased significantly after exposure to the "EDTA + DTT" ($p<0.05$), "EDTA + Tween-20" ($p<0.0001$), and "DTT + Tween-20" ($p<0.001$) cocktails (Fig. 7C). In contrast, the relative

number of flakes decreased significantly after exposure to the “Saline + DTT,” “Saline + Tween-20,” “EDTA + Tween-20,” and “DTT + Tween-20” cocktails ($p<0.0001$) (Fig. 7B).

“Triple cocktail” treatments tested combinations of three reagents against flake samples. After imaging, flakes appeared to increase in size for the cocktails “Saline + EDTA + Tween-20” and “EDTA + DTT + Tween-20” and decrease slightly for the cocktail “Saline + DTT + Tween-20” (Fig. 7A). No apparent size change was observed for the cocktail “Saline + EDTA + DTT” by imaging (Fig. 7A). Qualitative observations of changes in flake size were confirmed as flake area measurements reported significant increases after exposure to “Saline + EDTA + Tween-20” ($p<0.0001$) and “EDTA + DTT + Tween-20” ($p<0.0001$) cocktails (Fig. 7C). Flake quantification and analysis also determined that flake size decreased after exposure to the cocktail “Saline + DTT + Tween-20” ($p<0.05$) (Fig. 7C). This decrease in flake area was significant; however, the decrease was minimal with an average effect of 5.9%. Furthermore, relative number of flakes significantly decreased after exposure to the cocktails “Saline + EDTA + Tween-20” ($p<0.01$), “Saline + DTT + Tween-20” ($p<0.0001$), and “EDTA + DTT + Tween-20” ($p<0.0001$) (Fig. 7B).

A sample of flakes was exposed to the “quadruple cocktail,” which combined all four reagents: DTT + Tween-20 + Saline + EDTA. After imaging, flakes did not appear to change in size at 20X magnification (Fig. 7A). This was confirmed via size quantification and analysis of the flake population, which showed no significant changes in relative flake size ($p>0.05$) (Fig. 7C). In contrast, the relative number of flakes decreased significantly ($p<0.0001$) (Fig. 7B).

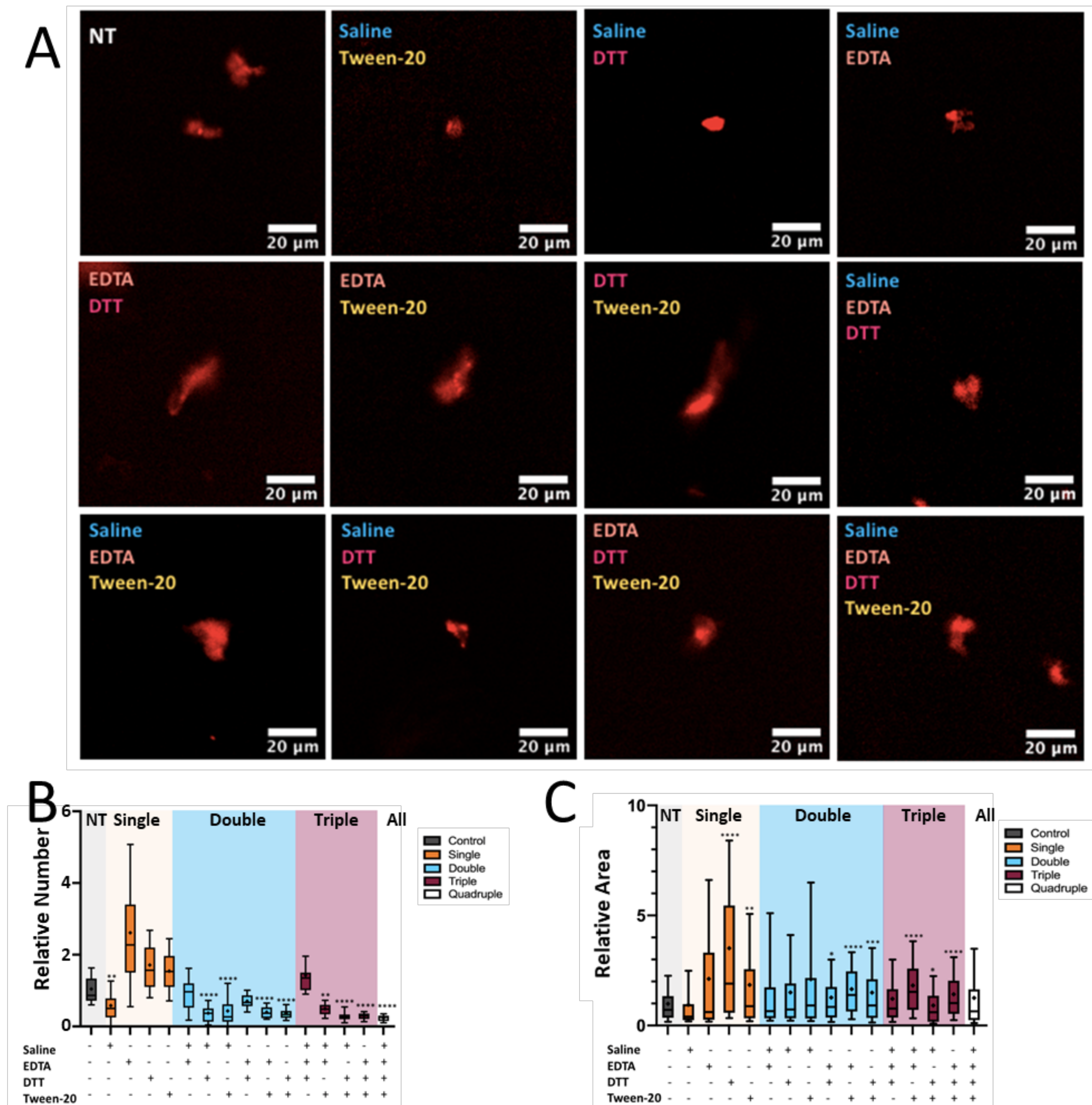

Figure 7. Mucus Flake Analysis after Cocktail Treatment (A) Mucus flakes treated with various combinations of saline (3%), EDTA (50 mM), DTT (10 mM), and Tween-20 (1%) and representative flake size (median area) of each cocktail displayed. Cocktails shown are labeled as “double” (treatment with two reagents), “triple” (treatment with three reagents), and “quadruple (treatment with four reagents).” (B) Mucus flakes treated with various combinations of saline (3%), EDTA (50 mM), DTT (10 mM), and Tween-20 (1%), and number of mucus flakes was analyzed after treatment. Cocktails labeled as “single,” “double,” “triple,” and “quadruple (All; EDTA, DTT, Tween-20, and saline).” (C) Mucus flakes treated with various combinations of saline (3%), EDTA (50 mM), DTT (10 mM), and Tween-20 (1%), and relative area of mucus flakes was analyzed after treatment. P-Values: *<0.05, **<0.01, ***<0.001, ****<0.0001. Plot whiskers measure 10-90 percentile error bars, and all p-values for each sample were measured against the control (NT, -Saline/EDTA/DTT/Tween-20). Population average represented as ”+” and median displayed as solid bar. Samples ran in triplicate where n>100. A lower bound of 1μm^2 was used to quantify.

V. Discussion

In CF, higher mucus flake number correlates with inflammatory and metabolic biomarkers of disease.[6] Larger flake size may be associated with CF disease as larger flake size suggests higher mucin quantity.[10] Increases in mucin quantity have been associated with abnormal mucus accumulation. To characterize flake disassembly by removing stabilizing cross-linking interactions, calcium chelator (EDTA), reducing agent (DTT), surfactant (Tween-20), and mucolytic (hypertonic saline) reagents were added to *de novo* flakes in various combinations.

Using a novel microfluidic-based flake synthesis, we made multiple observations by flake imaging and quantitative analyses that yielded insights into the nature of flake disassembly and the use of artificial flakes as a platform for screening potential CF treatments.

Flake size increased for all DTT concentrations tested; however, the size increase was only statistically significant for the 100 mM treatment (Fig 4). We speculate that the relative flake size increase at 100 mM is due to swelling in the flake structure. DTT forms a stable ring-structure with the disulfide bonds between mucin monomers, thereby eliminating this interaction, which causes mucin polymers to break apart and destabilize the flake as a result.[4] Subsequently, flakes may become more soluble and penetrable to water, which infiltrates the architecture, thus allowing swelling and hydration. As a result, swollen, post-DTT treated flakes may improve airway mucosal clearance if additional mucus-hydrating reagents are added as treatments.

After flake treatment with DTT, relative flake number statistically increased after 1 mM DTT treatment but was insignificant after 10 mM and 100 mM DTT treatment. The significant increase in flake number after exposure to 1 mM DTT may reflect the elimination of disulfide bonds between mucin monomers and cause flakes to crumble into multiple pieces, which correspond to smaller flakes compared to higher concentrations. Lower concentrations of DTT promote flake crumbling while higher concentrations of DTT promote flake swelling due to more complete reducing of mucin polymers. Future experiments will examine the dose dependency of flake number and size at a wider range of DTT concentrations to confirm this idea.

While higher flake number is associated with CF disease, the increase in flake number after DTT treatment coupled with flake swelling may indicate optimal conditions for airway mucosal clearance if translated to a clinical setting with an FDA approved reducing agent.[6] This is because as mucin monomer linking interactions are removed, flake swelling occurs and the structures become unstable and begin to break apart into flake fragments that are hydrated, thus allowing for more efficient airway mucosal clearance.

Previous literature describes the importance of calcium chelation during mucin granule exocytosis, which depicts the transition from a compact mucin network to an expanded form. [4] In CF, the secretion of bicarbonate that acts as both an alkalinizing and chelating agent is hindered and mucoviscidosis can occur as a result of abnormal post-secretion mucin unfolding.[4] Treatment of model CF mucus with EDTA was previously found to normalize CF mucus properties; however, the impacts of EDTA exposure on mucus flake structure have not yet been explored.[4] Calcium chelation after flake treatment with EDTA resulted in a significant increase in flake size and a significant decrease in flake number at all concentrations. The increase in flake size after EDTA treatment may be due to flake-swelling as calcium-mucin cross-linking is disrupted, thereby rendering the flakes more soluble in water. Our data also suggests that calcium chelation via EDTA promotes mucin polymer expansion within the flakes, thus resulting in significant increases in flake size. These results are supported by past literature findings that describe mucin expansion in healthy mucus when calcium ions are chelated by extracellular bicarbonate and are exchanged for sodium ions.[3,4]

While an increase in relative flake number was observed after DTT treatment, a decrease in relative flake number was reported after EDTA exposure. This may be attributed to the fact that EDTA targets calcium-mucin cross-linking rather than disulfide bond-mucin interactions. As a result, our findings may suggest that as EDTA chelates the calcium within flakes, mucin polymers—and thereby the mucus flakes—may destabilize, become hydrated, and expand, which leads to flake dissolution as flakes subsequently break apart and produce the observed decrease in flake number. Previous literature describes

higher flake number in CF disease, and thus calcium chelators may act as a potential treatment for flake disassembly as they caused flake swelling and flake dissolution after EDTA treatment.[6]

While the "single sweep" treatment of flakes with EDTA reported changes in flake number and size, these changes were not calculated to be significant due to the large size of error bars for both flake number and size. The large error bars and differences in significance observed in the EDTA "single sweep" treatment (Fig. 7) should be attributed to unpreventable batch-to-batch variability due to varying degrees of modification during mucin purification processes.

To investigate the impact of hydrophobic interactions removal on mucus flakes, multiple concentrations of the surfactant Tween-20 were tested. Significant increases in flake number were observed after flake treatment with 0.1%, 1%, and 5% Tween-20. These results suggest that the removal of hydrophobic interactions that occur between un-glycosylated protein regions of N and/or C termini and cysteine-rich segments of HBE mucin facilitate the disassembly of mucus flakes. This is because the removal of hydrophobic interactions within the flakes may lead to structural destabilization, thus causing the flakes to crumble into fragments, which flake analysis quantifies as an increase in flake number. The ability of Tween-20 to change flake number may demonstrate the potential for surfactant-based CF treatments.

In figure 7, the "single sweep" Tween-20 treatment findings appear to conflict with the single Tween-20 treatment findings from figure 6. Specifically, the "single sweep" Tween-20 treatment was observed to significantly increase flake size but insignificantly change flake number (Fig. 7). In contrast, figure 6 reports an insignificant change in flake size and a significant increase in flake number at the same concentration. These differences between the single Tween-20 treatment (Fig. 6) and the "single sweep" Tween-20 treatment (Fig. 7) can be attributed to batch-to-batch modifications in mucins used in flake synthesis as a result of unpreventable variability in the mucin purification process.

Maximization of mucus flake disassembly after treatment with Tween-20, EDTA, saline, and DTT was achieved in the treatment of flakes with double, triple, and quadruple cocktails of these reagents. The significant increase in flake size reported for the double ("EDTA + DTT," "EDTA + Tween-20," and

"DTT + Tween-20") and triple ("Saline + EDTA + Tween-20" and "EDTA + DTT + Tween-20") cocktails may indicate flake swelling as a potential underlying cause of flake size increases due to the combined effects of calcium chelation (EDTA), disruption of hydrophobic interactions (Tween-20), and disulfide bond reduction (DTT). It is suspected that this swelling is due to the disruption of the targeted biochemical interactions, thus rendering the flakes more permeable to water. Interestingly, significant changes in flake size were not observed for the "single sweep" saline treatment (Fig. 7) or the single treatments of DTT (Fig. 4) and Tween-20 (Fig. 6) at these same concentrations (3%, 10 mM, and 1% respectively). Thus, these findings suggest that saline, Tween-20, and DTT may function as flake disassembly agents in the presence of other reagents such as EDTA. For this reason, an "amplifying phenomenon" may explain these findings where an additive effect is created via the combination of reagents' distinct disassembly mechanisms. Subsequently, the distinct swelling and disassembly mechanisms of EDTA, saline, DTT, and Tween-20 are additive and amplified in the presence of each other.

Similarly, the decrease in number after flake exposure to double (Saline + DTT, Saline + Tween-20, EDTA + Tween-20, and DTT + Tween-20), triple ("Saline + EDTA + Tween-20," "Saline + DTT + Tween-20," and "EDTA + DTT + Tween-20"), and quadruple (Saline + EDTA + DTT + Tween-20) cocktails may demonstrate the "amplifying phenomenon." As a result, the combination of multiple treatments amplifies each reagent's individual flake disassembly properties, thus resulting in the observed widespread significant decrease in flake number. Specifically, the decrease in flake number may demonstrate the additive disruption of multiple types of interactions (calcium cross-linking for EDTA, hydrophobic interactions for Tween-20, disulfide bonds for DTT, and ionic bonds for saline), which may result in the dissolution and disassembly of flakes as reflected by a decrease in relative flake number.

Our results also demonstrate the possibility of "treatment negation" where treatments that were previously effective in single reagent exposures did not alter flake size or number in the presence of other compounds. Thus, one reagent may shield flakes from other reagents' therapeutic properties, thereby explaining the lack of significant changes in flake number or size observed. This may be the case for the

"Saline + EDTA" and "Saline + EDTA + DTT" cocktails where no significant changes in flake size or number were reported despite EDTA and DTT's previously observed flake disassembly properties in single treatments.

Our findings demonstrate that selected double cocktails (EDTA + Tween-20 and DTT + Tween-20) and triple cocktails (Saline + EDTA + Tween-20, Saline + DTT + Tween-20, and EDTA + DTT + Tween-20) prove to be the most effective cocktails. This is supported by the significant changes in both size and number observed for each cocktail, which may reflect flake swelling and flake dissolution as interactions between mucin polymers in flakes are disrupted, thus promoting maximum flake destabilization and disassembly. These cocktails caused changes in flake number via the dissolution of flakes which may revert CF mucus to a healthy state. In addition, the remaining flakes swelled, reflecting an increased possibility for airway mucosal clearance in CF. This contrasts with cocktails that only reported either a change in flake size or number but not both. A change in only flake size or number could subsequently limit a cocktail's effectiveness in flake disassembly as only one CF disease indicator (change in flake number or size) is affected. This is the case for the quadruple cocktail, which contained the highest number of treatments, yet only a change in flake number was observed. This cocktail may still promote flake dissolution but may not be the most effective cocktail to optimize flake swelling, and ultimately, airway mucosal clearance in diseased states.

To assess the translational capabilities of calcium chelators, surfactants, and reducing agents to clinical mucus flakes in CF patients, FDA-approved reagents should be tested using identical methods used in this study. For example, to translate DTT to clinical applications, the clinically-used reducing agent, N-acetyl cysteine, a thiol-based compound, should be tested.[4] To translate Tween-20 to clinical applications, the use of sodium bicarbonate, a current CF surfactant treatment, should be explored.[9] Currently, there are no FDA-approved calcium chelators for CF treatment; however, OligoG, a guluronate-rich alginate with a high affinity for calcium ions, is currently in phase II clinical trials for the calcium chelation-dependent treatment of CF patients.[4]

In this study, multiple limitations exist, which should be considered in future investigations. Conflicts between single treatment samples and "single sweep" treatment samples (i.e. same single reagent tested in two different flake samples) arose. These conflicts in findings were attributed to batch-to-batch variability due to mucin purification inconsistencies between separate mucin samples used for flake synthesis. While each treatment within a flake sample was tested in triplicate, the mucin variability between two separate flake samples reflects the mucin heterogeneity that is unavoidable due to limitations in mucin purification methodologies. Furthermore, the novel flake synthesis used in this study is limited by its production of heterogenous flakes in morphology and size that may result in significant variance between flake samples. To reduce the impact of this variance in a flake sample, each treatment was normalized to the non-treated control sample for both flake number and size measurements.

In summary, the synthesis and subsequent exposure of flakes to various combinations of reagents, such as a calcium chelator, a reducing agent, a surfactant, and a known mucolytic CF treatment, offer novel insights into flake disassembly. Upon assessing previous literature to understand the interactions between treatments and mucin polymers, changes in mucus flake size, number, and morphology can be understood on a biochemical level and the impact of such changes in CF treatment can be predicted. Our results reflect the use of artificial mucus flakes as a model for exploring flake disassembly. Specifically, our findings demonstrate the successful use of artificial flakes as a platform for testing therapeutics by analyzing flake disassembly methods. More importantly, this approach can be used to explore future therapeutic and translational pathways to disassemble flakes and reduce their burden in the airways in CF.

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